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Fructans from oat and rye: Composition and effects on membrane stability during drying

Dirk K. Hincha [a,*], David P. Livingston III [b], Ramaswamy Premakumar [b], Ellen Zuther [a], Nicolai Obel [a,1], Constança Cacela [a,2], Arnd G. Heyer [c]

[a] *Max-Planck-Institut für Molekulare Pflanzenphysiologie, D-14424 Potsdam, Germany*
[b] *USDA and North Carolina State University, 840 Method Road, Unit 3, Raleigh, NC 27695, USA*
[c] *Biologisches Institut, Abt. Botanik, Universität Stuttgart, Pfaffenwaldring 57, D-70569 Stuttgart, Germany*

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Abstract

Fructans have been implicated in the abiotic stress tolerance of many plant species, including grasses and cereals. To elucidate the possibility that cereal fructans may stabilize cellular membranes during dehydration, we used liposomes as a model system and isolated fructans from oat (*Avena sativa*) and rye (*Secale cereale*). Fructans were fractionated by preparative size exclusion chromatography into five defined size classes (degree of polymerization (DP) 3 to 7) and two size classes containing high DP fructans (DP > 7 short and long). They were characterized by high performance liquid chromatography (HPLC) and matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS). The effects of the fructans on liposome stability during drying and rehydration were assessed as the ability of the sugars to prevent leakage of a soluble marker from liposomes and liposome fusion. Both species contain highly complex mixtures of fructans, with a DP up to 17. The two DP > 7 fractions from both species were unable to protect liposomes, while the fractions containing smaller fructans were protective to different degrees. Protection showed an optimum at DP 4 and the DP 3, 4, and 5 fractions from oat were more protective than all other fractions from both species. In addition, we found evidence for synergistic effects in membrane stabilization in mixtures of low DP with DP > 7 fructans. The data indicate that cereal fructans have the ability to stabilize membranes under stress conditions and that there are size and species dependent differences between the fructans. In addition, mixtures of fructans, as they occur in living cells may have protective properties that differ significantly from those of the purified fractions.

Keywords: Desiccation; Fructan; Liposome; Oat (*Avena sativa*); Rye (*Secale cereale*)

1. Introduction

Fructans are polymers of fructose containing no or one internal or terminal glucose unit. They are found in many plant species throughout temperate climate regions (see [1] for a review) and are considered to serve primarily as storage carbohydrates [2] that can account for as much as 80% of the dry weight in specific tissues of some species [3].

The discovery that fructans accumulate in overwintering organs during cold acclimation [4] and also under dehydration stress [5,6] led to the speculation that fructans may also play a role in the protection of tissues under abiotic stress conditions. However, other studies [7–9] called the relationship of fructan content to stress tolerance into question, which indicates that such a relationship may be more complex than originally proposed [10].

In transgenic plants of perennial rye grass [11] and tobacco [12,13], the accumulation of fructans increased the tolerance of freezing and drought stress, pointing to a functional role of these oligosaccharides in plant stress tolerance. Since cellular membranes have been shown to be the primary targets of drought and freezing stress [14,15], a functional role for fructans

* Corresponding author. Tel.: +49 331 567 8253; fax: +49 331 567 8250.
E-mail address: hincha@mpimp-golm.mpg.de (D.K. Hincha).
[1] Present address: Landsberger Str. 30, D-80339 München, Germany.
[2] Present address: Instituto Superior Técnico, Centro de Química Estrutural, Av. Rovisco Pais, 1049-001 Lisboa, Portugal.

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in membrane stabilization would be required to attain protective effects. Because fructans are localized in the vacuole [16], but also in the apoplast under stress conditions [17], the tonoplast and plasma membrane would be likely targets for stabilization of plant cells by fructans.

The effects of sugars on membrane stability have been investigated extensively with liposomes as model systems (see [18] for a recent review). In these studies it has been shown that both linear plant inulins and a high-molecular-weight, branched bacterial levan are able to stabilize membranes during desiccation or freeze-drying [19–22]. In addition, it was shown that inulins become more effective in membrane stabilization during drying with increasing degree of polymerization (DP), while malto- and manno-oligosaccharides become less effective [19,21,23].

To help clarify the often contradictory relationship between fructan content and composition, and abiotic stress tolerance, we have isolated fructans from oat and rye, two cereal species that differ widely in their abiotic stress tolerance. For the oat cultivar Wintok and the rye cultivar Rosen used in our experiments, LT_{50} values (temperature of 50% survival after freezing) of −10.7 °C and −16.5 °C, respectively, have been reported [10]. We have compared the DP-dependent effects of these fructans on the stability of liposomes during drying and rehydration. These data define the physical capability of different cereal fructans to provide protection to membranes under dehydration conditions and give a first indication of the functional complexity of the highly branched cereal fructans as compared to the linear inulins.

2. Materials and methods

2.1. Plant growth

Oat (*Avena sativa*, cv Wintok) and rye (*Secale cereale*, cv Rosen) plants were grown in flats containing Metromix 550 soil medium with approximately 150 seeds per flat. They were grown at 13 °C day, 10 °C night temperature, with a 12-h photoperiod of 400 μmol of photosynthetically active radiation (PAR). After 5 weeks they were transferred to a growth chamber at 3 °C with 300 μmol of PAR and a 10-h photoperiod for 3 weeks to induce fructan accumulation. The plants were watered five times per week and fertilized twice weekly with a modified Hoagland solution [24].

2.2. Isolation and purification of oat and rye fructans

The isolation and purification procedure is based, with several modifications, on a method described previously [24]. Leaves and stems of cold acclimated plants were harvested in batches of approximately 300 g and ground in a blender with absolute ethanol for 60 s. The slurry was transferred to a beaker, placed in a boiling water bath for 20 min and then squeezed through cheese cloth into polypropylene centrifuge bottles.

The remaining plant material was re-ground in the blender for 60 s with water and squeezed through fresh cheese cloth into polypropylene centrifuge bottles. Both the water extract containing larger fructans and the ethanol extract containing simple sugars and primarily small fructans were centrifuged at 2500 RPM for 10 min. The supernatants were flash evaporated at 30 °C to dryness. From 100 g of fresh oat or rye leaves we obtained about 10.5 g of crude ethanol extract and 9.5 g of crude aqueous extract.

The dried extracts were dissolved in water to 200 mg/ml and protein was precipitated with 10% (w/v) lead acetate. Samples were heated to 90 °C for 15 min and centrifuged as above to remove precipitated proteins. This process was repeated approximately three to five times, until no more precipitate was visible.

To both water and ethanol extracts Dowex 1 anion exchanger (Sigma; activated with 2 M NaOH) was added while stirring continually, until the pH reached 8. Then Dowex 50w cation exchanger (Sigma; activated with 2 M HCl) was added to bring the pH to 7. The resins were allowed to settle, the extracts were decanted and the resins rinsed several times with water. The combined extracts were then filtered through activated Waters SEP-PAK C18 cartridges (Waters Corp. MA), clarified with charcoal and passed through Whatman #4 cellulose filters under vacuum. Extracts were then flash evaporated and stored under vacuum in a desiccator until use.

For column chromatography, dried extracts were dissolved in water to 500 mg/ml and filtered first with a 5-μm and then with a 0.45-μm filter. Two ml of extract were injected into a preparatory column (4 cm × 245 cm) packed with Toyopearl HW-40S (TOSOH Biosep LLC, Montgomeryville, PA) size exclusion resin. The column was eluted with water at a flow rate of 1.2 ml/min. The retention times of the collected fractions were: DP > 7 large: l9 h 5 min; DP > 7 small: 21 h 15 min; DP7: 22 h 50 min; DP6: 23 h 40 min; DP5: 24 h 40 min; DP4: 25 h 50 min; DP3: 26 h 40 min. Fractions were flash evaporated, lyophilized and stored under vacuum until further use.

2.3. Matrix-assisted laser desorption ionisation-time of flight mass spectrometry (MALDI-TOF MS)

Oligosaccharides were analyzed by a Voyager DE-Pro MALDI-TOF instrument (Applied Biosystems, Foster City, CA) using an acceleration voltage of 20 kV with a delay time of 350 ns. Mass spectra were obtained in the reflectron mode using 2,5-dihydroxybenzoic acid (10 mg/ml) as matrix. The relative abundance of the various oligosaccharides was calculated using a PERL based program as described before [25].

2.4. Chromatographic analysis of fructans

Fructans were analyzed by high performance liquid chromatography (HPLC) with a CarboPac PA-100 anion exchange column on a Dionex DX-500 gradient chromatography system (Dionex, Sunnyvale, CA) with pulsed amperometric detection. The column was equilibrated in 0.15 M NaOH and was eluted with a linear gradient from 0.15 M to 1.0 M Na-acetate as described previously [26]. For comparison, a standard mixture of sugars was prepared, containing glucose (Glc), fructose (Fru), sucrose (Suc) (all from Sigma), and inulins with a degree of polymerization of 3, 4 and 5 (Megazyme, Wicklow, Ireland).

2.5. Preparation of liposomes

Egg phosphatidylcholine (EPC) was purchased from Avanti Polar Lipids (Alabaster, AL). The lipid was dried from chloroform under a stream of N_2 and stored under vacuum over night to remove traces of solvent. Liposomes were prepared from hydrated lipids using a hand-held extruder with two layers of polycarbonate membranes with 100 nm pores ([27]; Avestin, Ottawa, Canada).

2.6. Leakage experiments

Carboxyfluorescein (CF) was purchased from Molecular Probes (Leiden, The Netherlands) and was purified according to the procedure described in [28]. Liposomes for leakage studies were made as previously described [29]. Briefly, an appropriate amount of EPC was hydrated in 0.25 ml of 100 mM CF, 10 mM TES, 0.1 mM EDTA (pH 7.4). After extrusion, the vesicles were passed through a NAP-5 column (Sephadex G-25; Pharmacia) equilibrated in TEN buffer (10 mM TES, 0.1 mM EDTA (pH 7.4), 50 mM NaCl), to remove the CF not entrapped by the vesicles. The eluted samples were then diluted with TEN to a lipid concentration of approximately 10 mg/ml. Liposomes (40 μl) were mixed with an equal volume of concentrated solutions of sugars in TEN and 20 μl aliquots were filled into the caps of microcentrifuge tubes. The samples were dried in desiccators at 28 °C and 0% relative humidity for 24 h in the dark [21]. Damage to the liposomes was determined as CF leakage after rehydration, with a Fluoroskan Ascent (Labsystems, Helsinki, Finland) fluorescence microplate

reader at an excitation wavelength of 444 nm and an emission wavelength of 555 nm [21].

2.7. Membrane fusion experiments

Liposome fusion after drying and rehydration as described above was determined using fluorescence resonance energy transfer [30] as described in detail before [29]. Briefly, two EPC liposome samples were prepared: one sample was labeled with 1 mol% each of the fluorescent probe pair N-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine (NBD-PE) and N-(lissamine Rhodamine B sulfonyl) dioleoyl-phosphatidylethanolamine (Rh-PE), both obtained from Molecular Probes (Leiden, The Netherlands), while the other sample was unlabeled. The two samples were combined after extrusion in TEN buffer in a 1:9 (labeled:unlabeled) ratio, resulting in a final lipid concentration of 10 mg/ml. The liposomes were mixed with concentrated solutes in the same manner as for the leakage experiments. Fusion was measured by fluorescence resonance energy transfer [30] with a Kontron SFM 25 fluorometer (Kontron Instruments, Neufahrn, Germany) at excitation and emission wavelengths of 450 nm and 530 nm, respectively.

3. Results

We have isolated total soluble sugars (mono-, di- and oligosaccharides) from the aerial parts of cold acclimated oat and rye plants. As a first step, we analyzed the composition of these preparations by HPLC and MALDI-TOF MS. These two methods yield complementary information. The retention times of sugars from the HPLC column depend both on DP and structural differences (e.g. branching) and the results are only semi-quantitative in the absence of appropriate standards for calibration. Time-of-flight mass spectrometry, on the other hand, gives quantitative information on the relative amounts of substances in the different size classes, but is completely insensitive to structural differences between the molecules within size classes. In addition, due to matrix effects, mono- and di- and trisaccharides are not reliably detectable in the MALDI-TOF MS spectra.

Fig. 1 and Table 1 show the HPLC and MS data, respectively, for the crude sugar fractions from oat and rye. It is obvious from comparison with the elution diagram obtained from the standard mixture, shown in the bottom panel of Fig. 1, that both preparations contain substantial amounts of mono-, di- and trisaccharides in addition to the higher DP fructans. Also, the peaks eluting before the monosaccharides indicate the presence of simple sugar alcohols. The nature of the peak eluting between Suc and DP3 (elution time approximately 8 min) is not known. Both species contain a highly complex mixture of fructans and both the HPLC and MS data indicate that rye contains a higher proportion of high DP fructans than oat.

For further analysis, these crude preparations were fractionated into seven different size classes by preparative size exclusion chromatography. Since low-DP sugars cannot be reliably analyzed by MALDI-TOF MS, we only analyzed these fractions by HPLC. The chromatograms (Fig. 2) show the expected stepwise shift of the peaks to higher retention times with DP but also a partial overlap in peak positions between DPs, which could in part be due to the presence of more than one DP fructan in some samples. This suggests that the single chromatographic step resulted in a substantial enrichment of the particular size class in any fraction, but not always in a complete purification. Therefore, throughout the paper, we call these fractions "size classes", because they are highly enriched in the nominal DP, but may also contain fructans of higher or lower DP.

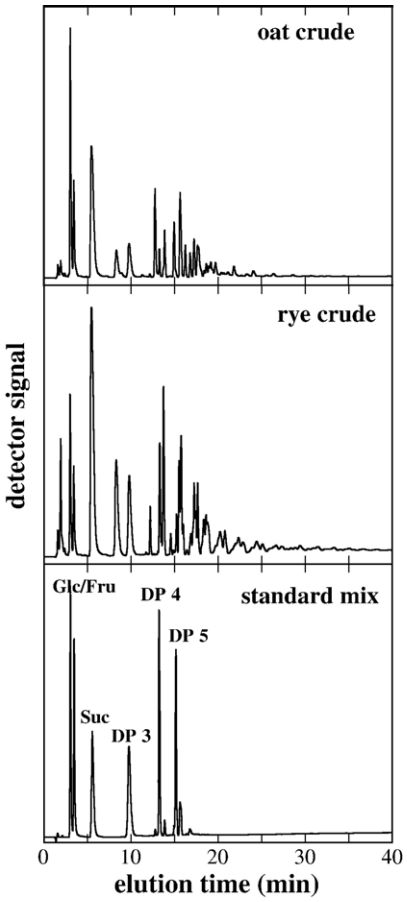

Fig. 1. HPLC analysis of crude sugar preparations from oat and rye leaves. For comparison, the analysis of a standard mixture of sugars (monosaccharides, disaccharide, and inulins with degree of polymerization (DP) from 3 to 5) is shown in the bottom panel. Longer elution times indicate a higher DP. A quantitative analysis of the size distribution of the fructans above DP 3 in these preparations is shown in Table 1.

In addition, peak overlap can result from structural differences, such as different glycosidic bonds and branching patterns, between fructans of the same DP, which would also lead to changes in retention time on the HPLC column. The structure of oat fructans of DP 3, 4 and 5 has been experimentally determined [31] and the elution patterns from the HPLC can be directly compared for some of them with the patterns shown in [26] and in the bottom panels in Fig. 2. The combination of these data indicates that the two peaks in oat DP 3 are 1-kestotriose and 6G-kestotriose, while the rye DP 3 fraction mainly contains 1-kestotriose and 6-kestotriose. The oat DP 4 consists of 1,1-kestotetraose (nystose), 1&6G-kestotetraose, 6G,1-kestotetraose, and 6G,6-kestotetraose [31]. The corresponding rye fraction contains mainly 1,1-kestotetraose (nystose) and 1&6G-kestotetraose. The oat DP 5 has been reported to contain seven different isomers [31] and a similar number of peaks can be discerned in Fig. 2 in our corresponding sample. Here, however,

Table 1
Size distribution (in % of total) of sugars in crude fructan preparations from oat and rye leaves, as determined by mass spectrometry

DP	Oat crude	Rye crude
4	26.2	17.2
5	22.7	16.5
6	18.9	14.4
7	13.2	12.0
8	8.3	9.2
9	4.5	6.6
10	2.7	5.8
11	1.8	3.6
12	1.6	3.8
13	n.d.	3.5
14	n.d.	2.7
15	n.d.	2.2
16	n.d.	1.2
17	n.d.	1.4

Note that mono-, -di, and trisaccharides cannot be resolved by this method (compare Fig. 1). No fructans of DP > 17 were detectable in these samples. (DP = degree of polymerization; n.d. = not detectable).

it is no longer possible to distinguish corresponding peaks in the rye sample. In general, at DP > 4 the peak distribution within a given size class is always wider for oat than for rye samples, indicating that oat fructans are structurally more heterogeneous than rye fructans.

In addition to these single DP fractions, we also obtained two fractions that consisted of higher DP fructans, labeled DP > 7 short (DP > 7S) and DP > 7 long (DP > 7L). It is obvious from the HPLC analysis that the size distribution is shifted to higher DPs in the long fractions, compared to the short fractions (Fig. 3). The chromatograms also indicate the presence of complex fructan mixtures of different DP and structure in all four samples.

Since these analyses showed compositional differences between the fructans from oat and rye, it was of interest to see, whether such differences would also be manifest at a functional level. We therefore investigated the ability of the sugars to stabilize liposomes during drying and rehydration. Fig. 4 shows that the crude sugar preparations from both oat and rye protected liposomes from leakage and fusion under our experimental conditions. However, protection against leakage was clearly inferior to that afforded by the well-known membrane stabilizer Suc, while protection against fusion was equal, or even slightly better, with the crude plant sugar preparations than with Suc. There were no clear differences in the effects of the preparations from the two different plant species.

The crude sugar preparations contain not only fructans, but also mono- and disaccharides (Fig. 1) that may mask differences between the fructans from oat and rye. We therefore investigated the lyoprotective effects of fructans in all seven isolated size classes separately. Again we used Suc (DP 2) as a reference. The results from these experiments (Fig. 5) revealed more complex patterns of protection, both within and between species. With the exception of the two DP > 7 fractions, all fructans from both species very efficiently protected liposomes against fusion during drying and rehydration.

This difference between the DP > 7 fractions and all other size classes was also apparent when leakage of a soluble fluorescent marker was determined instead of membrane fusion (Fig. 5; top panels). In addition, differences between size classes and between oat and rye fructans were clearly revealed. Within the oat fructans, the fractions with a nominal DP of 3, 4, and 5 showed the best protection and they were superior to all rye fructans. The rye fructans showed a weak optimum at DP 4, but

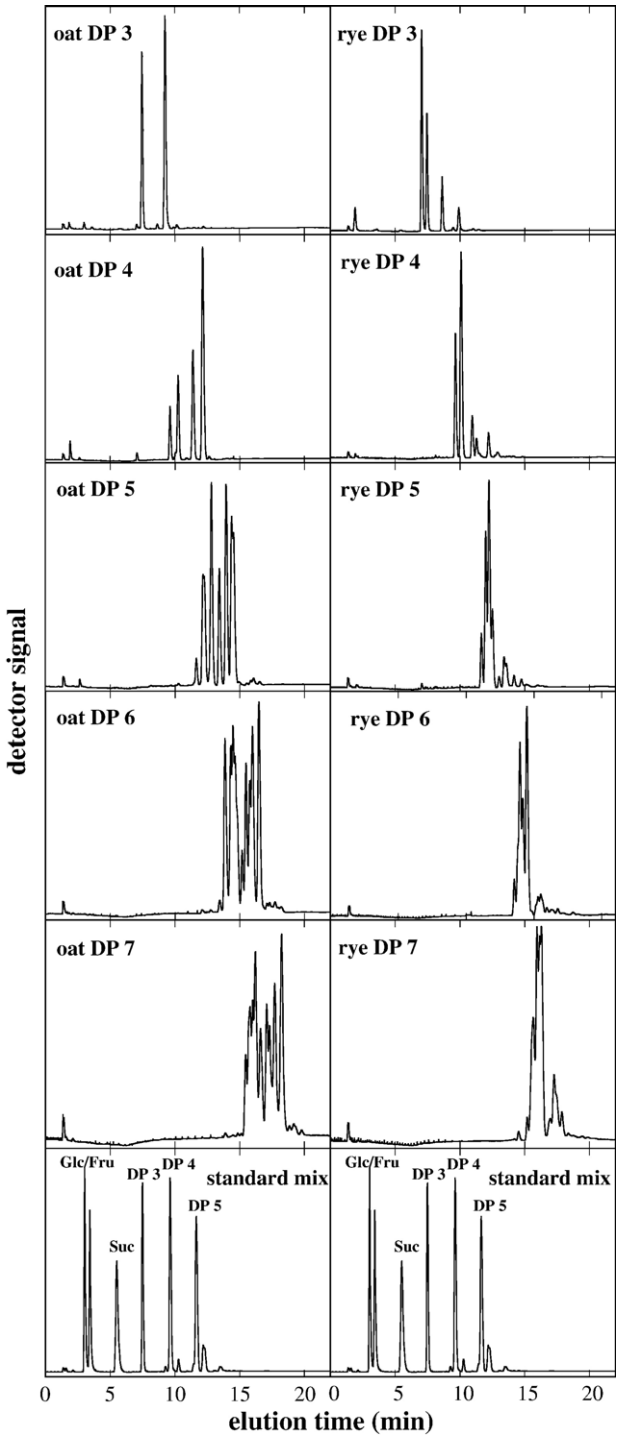

Fig. 2. HPLC analysis of fructan size classes purified from oat and rye leaves. See Fig. 1 for details.

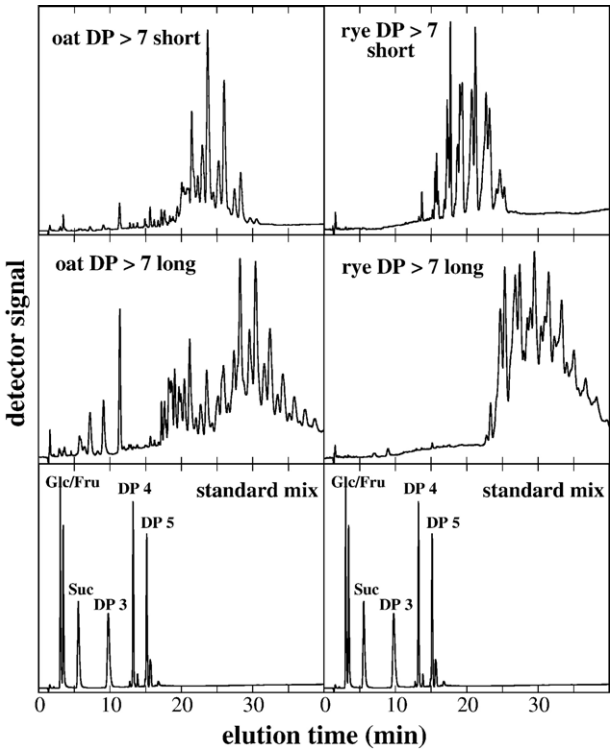

Fig. 3. HPLC analysis of two fructan size classes of high DP purified from oat and rye leaves. See Fig. 1 for details.

in general the differences between the different fractions were less pronounced in rye than they were in oat.

Since fusion of liposomes has been shown to be one of the causes of leakage, we investigated, whether the two parameters were correlated in these experiments as described previously for inulins [21], and found a clear linear correlation between leakage and fusion for both oat and rye fructans (data not shown). This indicates that the ability to prevent membrane fusion was an important factor in the protection of liposomes by cereal fructans.

Since solutes never occur in isolation in a cellular environment, it was of particular interest to see, whether the high DP fructans (DP > 7) have protective effects in combination with low DP fructans (DP 2–7). For this purpose, liposomes were dried in the presence of mixtures of either fructan DP > 7S or DP > 7L from oat or rye, and one of the smaller fructan fractions from the same species at a 1:1 mass ratio. Fig. 6 shows that in comparison to the pure high DP fructan, the mixtures were much better protectants. The generally higher effectiveness of fructans from oat in comparison to rye in protecting liposomes from leakage during drying and rehydration that was already found for the single size classes (Fig. 5) was also evident for the mixtures. Quite strikingly, these mixtures showed virtually no dependence of protection on DP (Fig. 6).

The differences between liposome stability in the dry state in the presence of all size classes of oat and rye fructans and their mixtures can be more clearly seen in Fig. 7, which compares CF leakage values from samples containing 20 mg sugar/ml. For comparison, the horizontal lines at the top of the figures indicate the level of CF leakage in samples containing only the high DP size classes (DP > 7S or L). It can again be clearly seen that protection by these long chain fructans is much poorer than it is by any other size class and that in general oat fructans afford better stability to liposomes during drying and rehydration than rye fructans.

The striking point in these figures, however, is the position of the CF leakage values obtained from samples containing the fructan mixes, as compared to the samples containing the single size classes. These data provide strong evidence for synergistic effects between short (DP 2–7) and long (DP > 7S/L) fructans in membrane stabilization. This is indicated by the fact that the 1:1 mixtures do not yield leakage values that are halfway between the samples containing the single short and long size classes, but instead show much lower leakage values. In some cases (e.g. oat DP 6 or 7 mixed with DP > 7S), the mixtures actually provided better protection than either of the components.

4. Discussion

While fructans accumulate in many grasses and cereals during cold acclimation concomitant with an increase in their freezing tolerance (see Introduction), their functional role in cellular stress tolerance is unresolved. Since membranes are the

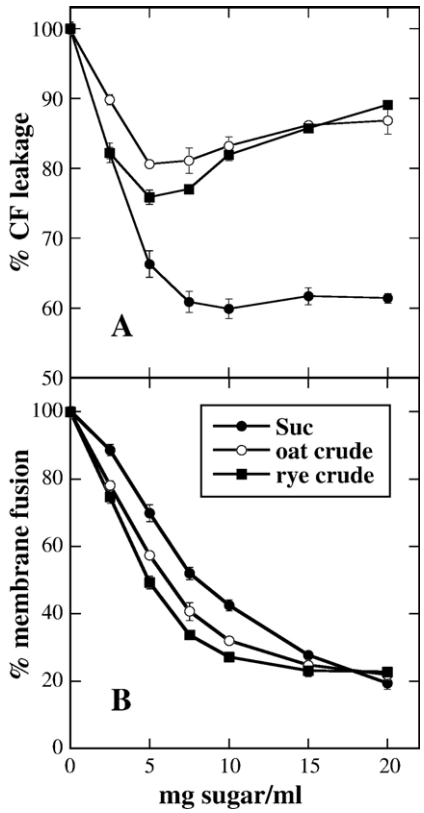

Fig. 4. Protection of large unilamellar liposomes from damage during drying and rehydration by different concentrations of Suc and crude sugar preparations from oat and rye leaves. For composition of these fructan fractions see Fig. 1 and Table 1. Membrane damage was assessed either as leakage of carboxyfluorescein (CF) from the vesicles (A) or as membrane fusion (B).

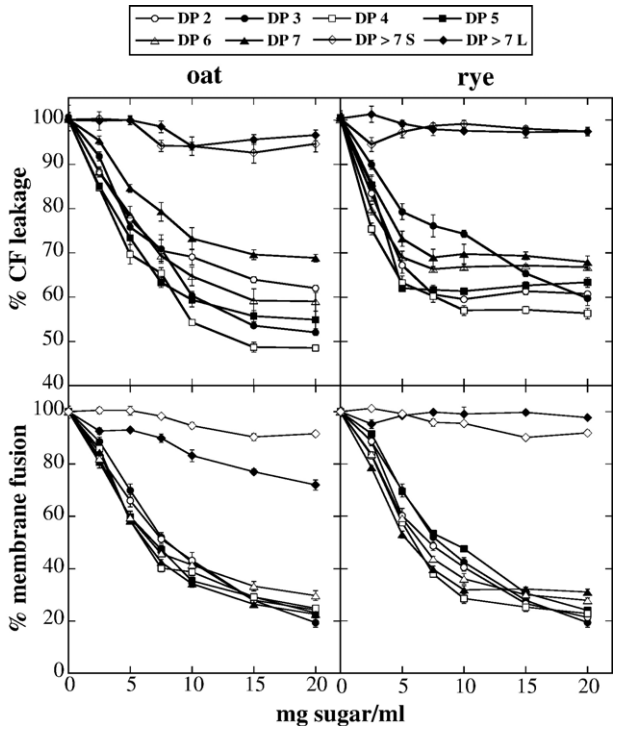

Fig. 5. Concentration dependent protection of large unilamellar liposomes from damage during drying and rehydration by different size classes of fructans purified from oat (left panels) and rye (right panels) leaves. For composition of these fructan fractions see Figs. 2 and 3. DP2 denotes sucrose. Membrane damage was assessed either as leakage of carboxyfluorescein (CF) from the vesicles (top panels) or as membrane fusion (bottom panels).

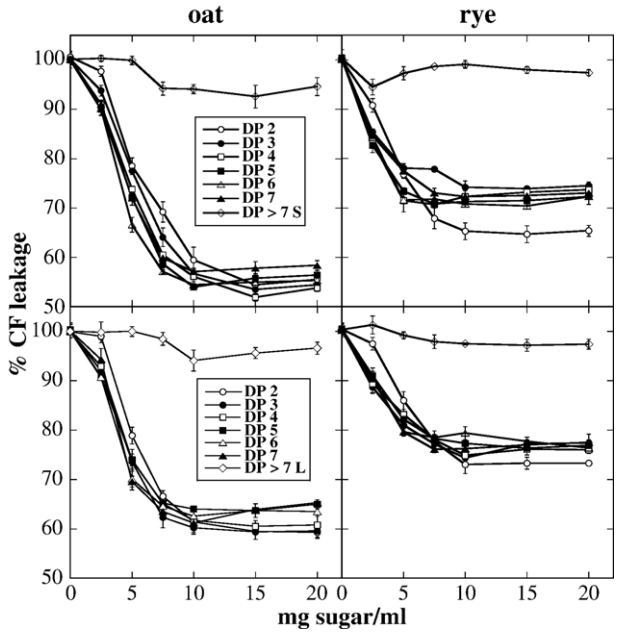

Fig. 6. Effects of fructan mixtures on liposome stability during drying and rehydration. The fractions DP > 7S (upper panels) or DP > 7L (lower panels) from oat (left panels) or rye (right panels) were mixed with all other size classes from the same plant in a 1:1 mass ratio (i.e. DP2 + DP > 7S; DP3 + DP > 7S etc.). For comparison, the effects of the pure DP > 7S or DP > 7L fractions is also shown (compare Fig. 5).

primary targets of abiotic stresses such as freezing and desiccation, liposomes have been extensively used as model systems to investigate the function of protective solutes such as sugars (see [18] for review). Damage to liposomes during drying and rehydration can be conveniently determined as the leakage of a soluble fluorescent marker such as CF from the vesicles. This leakage can be greatly reduced by the presence of sugars in the samples. Detailed investigations, mainly with the disaccharides Suc and trehalose, have established two general mechanisms that contribute to protection (see [18] for review).

On the one hand, leakage is triggered by lipid phase transitions. When membranes are dehydrated, this leads to an increase in the gel to liquid–crystalline phase transition temperature (T_m). In the case of EPC membranes, T_m increases from −5 °C in the fully hydrated state to 40 °C in the dry state [32], leading to a lyotropic phase transition from the liquid–crystalline to the gel state during drying at 28 °C, as performed in the present study. During rehydration, this transition is reversed. This leads to the leakage of soluble content from the liposomes, due to inhomogeneities in the membrane during the phase transition [33]. The presence of sugars prevents the dehydration-induced increase in T_m and therefore the phase transitions and the associated leakage. This is achieved through H-bonding of the sugar OH-groups to the lipid headgroups, thereby replacing the water of hydration (see [18,34] for reviews).

In addition to phase transitions, liposome fusion results in leakage [35]. Fusion can be prevented by vitrification (glass formation) of the suspending medium during drying, because it effectively stops diffusion and therefore the close approach of vesicles necessary for fusion. Since most sugars vitrify during drying at ambient temperatures [36,37], they are good protectants against liposome fusion and the associated leakage (see [18,34] for reviews).

While most studies on the protective effects of sugars on membranes during drying have been conducted with disaccharides, there are some published reports on different structural

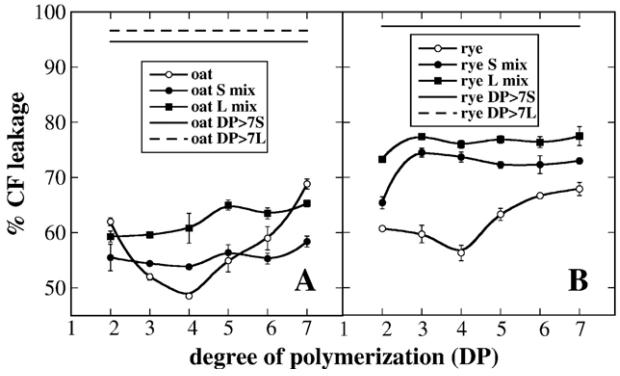

Fig. 7. Dependence of CF leakage from liposomes during drying and rehydration on the DP of the different size classes of fructans from oat (A) and rye (B). DP2 denotes sucrose. All samples contained 20 mg/ml of the respective sugars (compare Figs. 5 and 6). Samples contained either the pure size classes (open circles), or the mixtures of the different size classes with the DP > 7S (solid circles) or DP > 7L (solid squares) fractions. For comparison, the CF leakage values of the pure DP > 7S and DP > 7L samples are indicated by the horizontal lines at the top of the figures (same value for DP > 7S and L in B; compare Fig. 5).

families of oligosaccharides. It has been shown that inulins [19,21] and raffinose-family oligosaccharides [38] up to DP 5 show increased protection with increasing DP, while malto-oligosaccharides (up to DP 7) and manno-oligosaccharides (up to DP 6) show reduced protection with increasing DP [19,21,23]. This behavior has been related to the differential ability of the sugars to interact with the lipid headgroups in the dry state and thereby depress T_m and prevent lyotropic phase transitions [19,21,38].

The DP dependence of the protective effects of the fructans from oat and rye investigated in this study falls between these two groups. While protection against leakage increased up to DP 4, it decreased from DP 4 to DP 7. Since we found no DP dependence of the protection against membrane fusion, we can assume that all fructans from oat and rye up to DP 7 are good glass formers. This leads to the hypothesis that at a DP higher than 4 the ability of the sugars to H-bond to the lipid headgroups in the dry state and depress T_m is reduced. It should, however, be noted that for the oat fructans, the DP 6 fraction protected the liposomes at least as good as Suc and even the DP 7 fractions from both species still provided clear protection. This indicates a substantial interaction with the lipids even for the higher DP size classes. In comparison, the malto-oligosaccharide DP 7 and the manno-oligosaccharide DP 6 hardly provide any protection against leakage for EPC liposomes during drying [19].

It is also interesting to note that the DP 3, 4, 5, and 6 fractions from oat provided much better protection against CF leakage than the corresponding fractions from rye. This difference is not related to differential contamination of these fractions with sugars of a lower DP (compare Fig. 2). Therefore, these species-specific differences may be related to the presence of different isoforms, e.g. different branching patterns. Further purification and fractionation of these size classes will be necessary to resolve this question.

The DP > 7 fractions from either oat or rye hardly provided any protection to the liposomes during drying and rehydration. Since this was true for both leakage and fusion measurements, it is tempting to speculate that these high DP fructans are poor glass formers. Analogously to what we found for chicoree root inulins previously [21], this may be due to low solubility leading to precipitation of the high DP fructans from solution during slow drying.

This lack of a protective effect for DP > 7 fructans agrees with a lack of correlation between freezing tolerance and the amount of DP > 6 fructans in different oat accessions [39]. On the other hand, it was found that among eight wheat, one triticale and three rye cultivars the fructan fraction that was most highly correlated with freezing tolerance was the DP > 6 fraction, while the smaller size classes had the lowest correlation [9]. Among winter cereal crops (rye, wheat, barley and oat), oat has a unique fructan composition. The other cereals accumulate primarily large (DP > 7) fructans, while oat accumulates mostly smaller (DP 3–6) oligomers [24]. This fact is also evident in our analysis of the fructan composition of oat and rye (Fig. 1 and Table 1). In addition to its composition, the structure of fructans in oat is unique, because they are composed of a branched series of polymers based on neokestose which results in a non-terminal glucose molecule [40,41]. The extent of branching in oat fructans falls between the more linear fructans of grasses and the more branched structures of rye [41].

To the best of our knowledge, the effect of branching in carbohydrates on their ability to stabilize liposomes during drying and rehydration and to interact with membrane lipids in the dry state has never been investigated. From our earlier work on linear fructo-, malto-, and manno-oligosaccharides we hypothesized that structural flexibility of the sugar rings and the glycosidic linkages may be crucial determinants of the ability of oligosaccharides to interact with membrane lipids [19]. A recent systematic molecular dynamics simulation study has shown that all possible glycosidic linkages between two glucose molecules have characteristic flexibility patterns [42]. It is therefore not possible to *a priori* predict the properties of oligosaccharides containing combinations of different numbers of different glycosidic linkages. Further experimental and modeling studies will be necessary to resolve this complex and challenging problem.

Interestingly, when the DP > 7 fractions were mixed at a 1:1 mass ratio with lower DP fractions, protection improved dramatically in comparison with the DP > 7 fractions. In some cases, protection even exceeded that provided by the lower DP fractions alone, indicating specific synergistic effects.

Such effects have, to the best of our knowledge, not been reported in the literature before. The mixing of other low molecular weight protectants with Suc only resulted in a reduction in CF leakage intermediate between the two pure substances [43]. Only a mixture of the monosaccharide glucose with the polysaccharide hydroxyethyl starch provided protection superior to either component [44]. In this case, however, neither glucose nor the polymer provided any protection on their own, which is clearly different from the situation described in this study. Further detailed spectroscopic investigations will be required to elucidate the physical basis of the synergistic effects observed here with oat and rye fructans.

In addition to changes in fructan content, the content of many other solutes in plant cells changes during cold acclimation, along with changes in protein and lipid composition and many of these changes are tissue and cell specific [10,45,46]. These compositional differences could produce varying levels of synergistic effects between different solutes within a single plant. In this regard it is interesting to note that the crude sugar preparations from both oat and rye provided less protection to liposomes than the isolated short fructan fractions. In addition, higher concentrations of the crude preparations led to an increase in CF leakage. These results suggest that the crude preparations contained membrane destabilizing contaminants in addition to stabilizing sugars that significantly reduced the protective effects of the sugars. Obviously, we have just begun to characterize the functional properties of cellular components and their possible interactions. This complexity is also illustrated by the fact that in our experiments the fructans from the less freezing tolerant species (oat) provided better protection to liposomes during drying than the fructans from the more freezing tolerant species (rye). Further studies will have to show whether this is due to the particular cellular environment in the two species (e.g.

membrane composition or interactions with other soluble compounds) or whether the fructans show differential protection during drying and freezing.

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